BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Photolysis of the cyanide adduct of ferrous horseradish peroxidase

Brigitte Meunier [1], Peter R. Rich [*,1]

Glynn Research Foundation, Bodmin, Cornwall PL30 4AU, UK

Received 8 August 1996; accepted 1 October 1996

Abstract

The mutual interplay between redox- and anion-linked protonation processes in HRP has been investigated. Above pH 7.5, the midpoint potential is pH-independent and the lack of pH-dependency of the dissociation constant, K_d, of the ferrous HRP-cyanide compound shows that both the cyanide anion and the proton are stably bound in the product. Below pH 7.5, K_d increases with decreasing pH and the midpoint potential of the unligated form becomes pH-dependent. These data show that the redox- and anion-linked protonations are mutually exclusive, and are most consistent with protonation of the same residue in the distal heme pocket, His-42, in both cases. Photolysis of the ferroheme-cyanide compound has been investigated and conditions have been identified in which cyanide photolysis is accompanied by the co-migration of a proton, presumably from the protonated His-42. At room temperature, cyanide recombination from solution is a simple second order process with no observable geminate processes occurring on time scales slower than microseconds. However, a dramatic decrease in photolysis yield as temperature is lowered suggests that submicrosecond geminate recombination processes can become dominant. The ferroperoxidase-cyanide system provides a model system for study not only of movement of a more hydrophilic ligand through the protein structure, but also of its associated co-migrating proton.

Keywords: Horseradish peroxidase; Cyanide; Photolysis; Redox potential

1. Introduction

Ligand photolysis and recombination in hemoproteins and model heme compounds has been studied intensively. The myoglobin-CO compound has received particular attention. The complexity of CO recombination was first demonstrated by Austin, Frauenfelder and colleagues in studies with frozen media at low temperatures [1,2]. A four state model could be used successfully to describe the room temperature recombination behaviour of CO, NO, oxygen and alkyl isocyanides [3,4]. In this model, at least after heme thermal relaxation [5], photolysis results in an initial geminate state in which the iron atom and ligand are still close to van der Waal's contact distance (termed the 'pocket' or 'contact pair' intermediate). After several tens of picoseconds, the ligand moves away into the heme pocket to form a set of 'matrix' or 'protein separated pair' intermediates. The ligand can then reform the contact pair or move out into solution from which subsequent recombination can be very slow (nanoseconds to seconds). This type of model has been investigated

Abbreviations: HRP, horseradish peroxidase; K_d, dissociation constant; k_{obs}, observed rate constant.

[*] Corresponding author: Fax: +44 1208 821575; +44 171 3807096.

[1] Present address: Department of Biology, University College London, Gower street, London WC1E 6BT, UK.

PII S0005-2728(96)00140-5

by studying pathway dependencies on temperature, viscosity and pressure [3,4,6], by molecular dynamics simulations of ligand trajectories and protein motion [7], and by more physical considerations [8]. In addition, the possibility of engineering myoglobin by site-directed mutagenesis has allowed the roles of individual amino acids to be probed [9].

Horseradish peroxidase (HRP) is another hemoprotein of potential use as a model system for such studies. Although a high resolution structure of HRP is not yet available, the structure around its heme can be predicted based on the homology with other peroxidases [10] such as cytochrome-*c* peroxidase [11], lignin peroxidase [12] and the highly homologous peanut peroxidase [13], all of whose atomic structures have been determined. The structure of HRP itself has also been solved recently and should be available soon. Furthermore, site-directed mutants of the key catalytic residues in HRP have been produced [14–16]. Photolysis and recombination of the CO compound of HRP is well documented [17,18] but has not been intensively studied. In contrast to myoglobin, the room temperature photolysis quantum yield is less than 1 [19] and CO recombination from solution is much slower ($k = (3–4) \times 10^3$ M^{-1} s^{-1} at pH 7[18]). The lower photolysis yield at room temperature can be attributed to a picosecond geminate recombination process the extend of which can be increased further by binding of the substrate benzohydroxamic acid [20]. Studies of CO photolysis at low temperatures [21] indicate three separate processes of internal CO recombination, all faster than the single process seen in myoglobin, and suggest that slow recombination from solution can be attributed to a very low partition coefficient from solvent into the heme pocket.

The photolysis behaviour of ferrous heme–cyanide adducts is less well documented. In the case of myoglobin, the dissociation constant of this compound is very high [22] so that it can only be studied as a transient species. However, the cyanide compound of ferrous HRP has a K_d of around 1 mM at alkaline pH [23], making it more accessible to photolysis studies [24]. Since binding of cyanide is associated with the co-binding of a proton, at least at high pH [23], photolysis of the cyanide into solution should be accompanied by the loss of the same proton [25], hence making feasible the study of laser-induced protonic changes, in contrast to photolysis of the electroneutral ligands CO, NO, O_2 and alkyl isocyanides. In this paper we examine the involvement of protonation in cyanide binding and photolysis and its relation to well-known redox-linked protonation chemistry [26].

2. Materials and methods

2.1. Enzyme

Horseradish peroxidase (EC 1.11.1.7) isozyme C (HRPC) was purchased from Sigma (Type VI) and was used without further purification. Deazaflavin (7,8-nor, 3-methyl 5-deazalumiflavin) has been generously supplied by Prof. V. Massey, University of Michigan, USA.

2.2. Optical measurements

Difference spectra and transient kinetics at individual wavelengths were monitored with a single beam scanning/kinetic instrument built in-house (Glynn Research Enterprises, Bodmin, UK), and equipped with a liquid nitrogen cryostat (Oxford Instruments, Oxford) for measurements of transients at low temperatures. Short actinic light pulses (10 ns halfpeak width, 532 nm, $>$ 100 mJ/pulse) were provided by a frequency-doubled Nd-YAG laser (Spectron, Rugby, UK). For increased photolysis, longer light pulses were provided by an unfiltered commercial photographic flashlamp (Braun external linear xenon flashlamp model EF 300, 125 V working voltage, 1 ms halfpeak width). Transients were detected with a photomultiplier protected by appropriate combinations of filters. Photolysis transients were recorded individually at several wavelengths and kinetic spectra at various times after the flash were reconstructed from these transients as described previously [27].

2.3. Preparation of reduced, cyanide- and CO-ligated HRP

HRP was reduced by incubation with sodium dithionite in a buffer of 0.1 M potassium phosphate at the required pH. Cyanide-ligated enzyme was formed by addition of neutralised potassium cyanide. CO-

ligated enzyme was formed by bubbling with CO for 2–3 min after which time the cuvette was sealed to avoid the loss of CO.

At pH values below 7, an alternative technique using a flavin photoreductant [28] was adopted in order to prepare a stable sample of fully reduced, cyanide-ligated enzyme. This need arose because of the low midpoint potential of the cyanide adduct and the decreased reducing ability of sodium dithionite at low pH [29], leading to samples which tended towards a mixture of both oxidised and reduced, cyanide-ligated forms (see Section 3). HRP was dissolved to a final concentration of 2 μM in 0.5 ml of 0.2 M potassium phosphate, 10 mM EDTA, 10 mM glucose, 100 U/ml glucose oxidase, 500 U/ml catalase and 15 μM deazaflavin (7,8-nor, 3-methyl 5-deazalumiflavin). Neutralised potassium cyanide was added and the mixture was kept anaerobic with a positive pressure of argon above the liquid surface. The cyanide-ligated HRP was reduced by the deazaflavin radical [28] generated by 10–20 s illumination using white light from a standard projector lamp. Full formation of the reduced cyanide-ligated enzyme was established by monitoring the Soret band peak position change from 390 nm to 460 nm.

2.4. Potentiometric titrations

Anaerobic redox titrations were carried out in 2.5 ml of 0.1 M potassium phosphate at different pH values. HRP was added to a final concentration of around 2 μM. The buffer was kept anaerobic with a positive pressure of oxygen-free nitrogen above the liquid surface. Redox mediators were (E_m in mV, concentration in μM): 2-hydroxy-1,4-naphthoquinone (-220, 10), anthraquinone-2,6-disulfonate (-184, 10), anthraquinone-2-sulfonate (-250, 10), benzyl viologen (-311, 10) and methyl viologen (-455, 10). 50 mM sodium dithionite and 10 mM ferricyanide were used as reductant and oxidant, respectively. Redox potentials were measured in stirred samples with a platinum electrode and a Ag/AgCl reference electrode. For each measurement, stirring was stopped and spectra were taken from 390 to 460 nm. Potential was recorded at the start and the end of the scans and absorbance changes were plotted versus the average of the two potential readings. Heme reduction was monitored by a triple wavelength measurement at $440 - (430 + 450)/2$ nm. The triple wavelength measurements minimised interference from redox dyes or baseline drifts.

3. Results

3.1. Kinetics of recombination of cyanide after flash photolysis at pH 8.5

Ferrous HRP binds cyanide with a K_d of approx. 1 mM at alkaline pH [23] and binding is associated with a characteristic shift in the Soret band [30]. The binding spectrum caused by the shift is illustrated by the (reduced plus cyanide) minus (reduced) difference spectrum shown in Fig. 1A and has a peak and trough at 432 nm and 448 nm, respectively. Like the CO-compound, the ferroheme-cyanide compound is photolabile [24]. The photolysis and recombination kinetics at pH 8.5 in the presence of 10 mM cyanide and measured at 432–448 nm are shown in Fig. 1B. The initial yield of the photoproduct on this timescale using a short duration (10 ns) laser pulse was around 40%. A yield of 60% was obtained under comparable conditions for the CO-compound. However, cyanide and CO recombination are relatively slow, so that longer duration flashes can be used to obtain improved photolysis yields. With our unfiltered xenon flash of 1 ms halfpeak width, photolysis yields of 80–90% for the cyanide-compound and $>95\%$ for the CO-compound could be obtained at room temperature.

The kinetics of cyanide recombination after photolysis were slightly biphasic with around 80–90% fast phase and 10–20% slower phase. Similar biphasic decays were also observed when the binding of cyanide to ferrous HRP was monitored in the dark by manual addition of cyanide to unligated enzyme (data not shown). In the work below, we describe only the behaviour of the dominating fast component. The spectral changes and responses to experimental variables of the slower component appeared to be identical to those of the faster phase, and it seems most likely that this slower form reflects a minor heterogeneity of our HRP sample.

Spectra at various times after photolysis were constructed from transients taken at different wavelengths (Fig. 1A). The constancy of the isosbestic

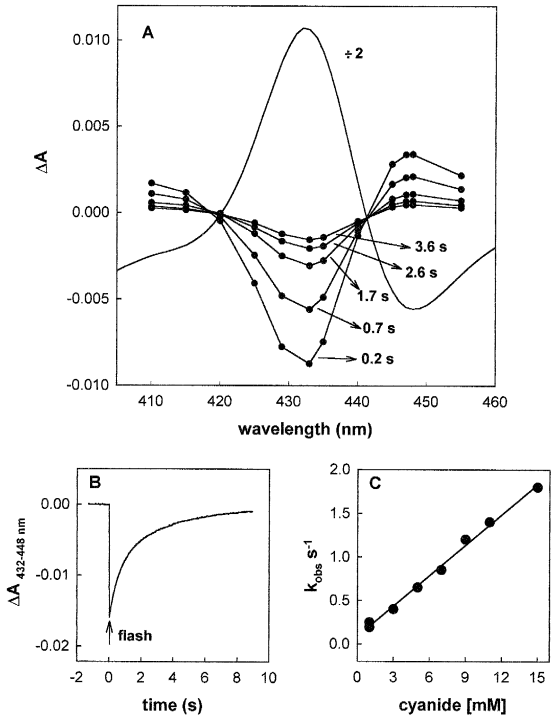

Fig. 1. Spectra and kinetics of cyanide recombination after photolysis. HRP was dissolved in 0.5 ml of 0.1 M potassium phosphate at pH 8.5 to a final concentration 0.8 μM. For the static binding spectrum (Panel A, solid line), the sample was reduced with solid dithionite and a baseline was recorded. The difference spectrum was recorded after addition of 10 mM neutralised potassium cyanide. For photolysis and recombination, identical samples with 10 mM potassium cyanide were used. Laser-induced transients were recorded at different wavelengths. The photolysis spectra at different times after the flash were reconstructed from them (Panel A). Panel B shows photolysis and recombination monitored at 432–448 nm. The observed rate constants (k_{obs}) were obtained by fitting an exponential decay to the transient trace. This measurement was repeated at different concentrations of cyanide. A plot of k_{obs} versus cyanide concentration is shown in panel C. The *y*-axis intercept and slope give k_{off} and k_{on}.

points (at 420 and 441 nm), maxima (at 448 nm) and minima (at 432 nm) showed that only a single optical component was present, and the transient spectra were the exact inverse of the static binding spectrum.

Exponential decays were fitted to the kinetics transients at different concentrations of cyanide. The observed recombination rate constants (k_{obs}) increased linearly with cyanide concentrations (Fig. 1C). The slope and intercept of the plot of k_{obs} versus cyanide concentrations gave values of $k_{on} = 115\ M^{-1}\ s^{-1}$, $k_{off} = 0.1\ s^{-1}$ and $K_d = 0.9$ mM.

3.2. Kinetics of recombination of cyanide after flash photolysis at pH 6.5 in the presence of dithionite

The photolysis behaviour of the cyanide-adduct was monitored at pH 6.5 in a sample of HRP which had been reduced by addition of solid dithionite in the presence of cyanide. The photolysis spectra are shown in Fig. 2B. Two optical components were observed with different relaxation kinetics. The immediate photoproduct corresponded to the inverse of the static spectrum of binding of cyanide to the fully reduced form at pH 8.5 (compare the transient spectrum at 0.2 s with the static binding spectrum at pH 8.5). This transient spectrum relaxed with a rate constant of around 2 s^{-1}, independent of the cyanide concentration, to a component which was the inverse of the apparent binding spectrum at pH 6.5 (Fig. 2A). Subsequently, this spectrum relaxed back to the dark state monophasically with a rate constant which increased linearly with the concentration of cyanide.

In order to test whether the complex kinetics were

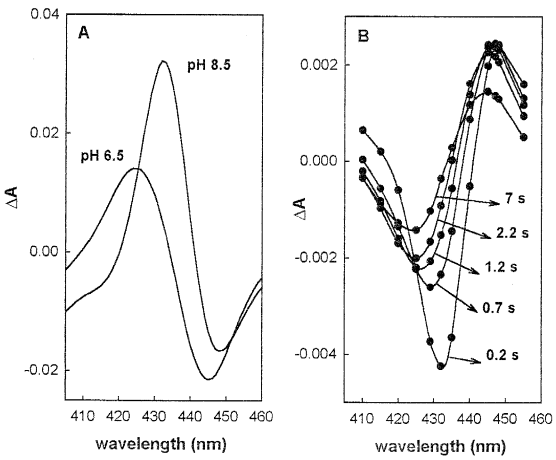

Fig. 2. Apparent binding spectra of cyanide to ferrous HRP and photolysis behaviour at pH 6.5 and 8.5. HRP was dissolved in 0.5 ml of 0.1 M potassium phosphate at pH 6.5 or at pH 8.5 to a final concentration 0.8 μM. The sample was reduced with solid sodium dithionite and a baseline was recorded. Panel A compares apparent static binding spectra on addition of 10 mM potassium cyanide. Panel B shows the transient spectra of the pH 6.5 sample after photolysis with a laser flash.

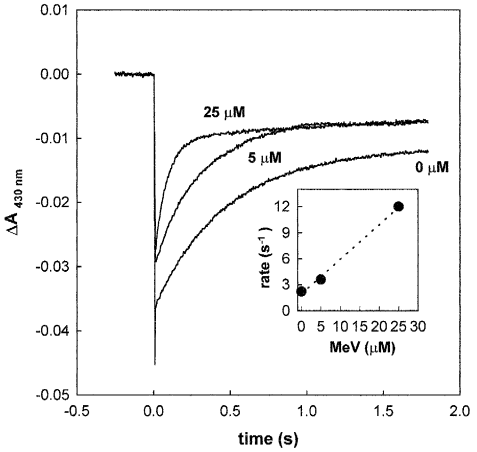

Fig. 3. Effect of methyl viologen on transient photolysis spectra at pH 6.5. A dithionite-reduced, cyanide ligated sample of HRP at pH 6.5 was prepared as described in Fig. 2. Methyl viologen was added to a final concentration of 5 or 25 μM and xenon flash-induced transients were recorded at 430 nm. Apparent decay rate constants of the fast component are plotted versus concentration of methyl viologen (inset).

due to post-photolysis redox changes of the heme group, we examined the effect of the redox mediator methyl viologen on the kinetics. These kinetics were most easily monitored at 430 nm, a wavelength at which both kinetic phases have large and roughly equal absorbance changes. As shown in Fig. 3, the rate of relaxation of the fast component increased with increasing concentrations of the redox mediator.

We interpret this biphasic behaviour in terms of the inability of sodium dithionite to keep the cyanide adduct of HRP in the fully reduced form at low pH, caused primarily by the pH-dependency of the midpoint potential of dithionite solutions [29]. By addition of solid dithionite at pH 6.5, the sample was kept around -400 mV, a potential which is close to the midpoint potential of cyanide-ligated HRP under these conditions (see later). In such a mixture, cyanide can be photolysed only from the ferrous form, hence giving an initial photoproduct which has a spectrum corresponding to the inverse of the binding spectrum of cyanide to the ferrous form. However, because of the presence of ferric-cyanide enzyme, a subsequent re-equilibration occurs in which some of the ferric-cyanide form is reduced to ferrous-cyanide form in order to re-establish the equilibrium position. This occurs with a rate constant of around 2 s^{-1}, independent of the cyanide concentration, but can be increased by the presence of a redox mediator. On longer timescales, slow recombination of the cyanide from the medium occurs to regenerate the original dark mixture of states.

3.3. Kinetics of recombination of cyanide after flash photolysis at pH 6.5 using deazaflavin as reductant

Because of the difficulties in maintaining a sufficiently low redox potential with sodium dithionite at low pH, another reductant had to be used in order to fully reduce the cyanide-ligated HRP at acidic pH. The only system which we found to be successful in achieving a sufficiently low potential even at low pH values was the photogenerated radical of a deazaflavin as described in [28]. When cyanide was added to the fully reduced HRP prepared at pH 6.5 in this way, it was found that the (reduced plus cyanide) minus (reduced) difference spectrum at pH 6.5 was virtually the same as that at pH 8.5, contrary to the data in Fig. 2A. Furthermore, the transient spectra after photolysis now showed only a single optical component which was the inverse of the static cyanide binding spectrum recorded at the same pH.

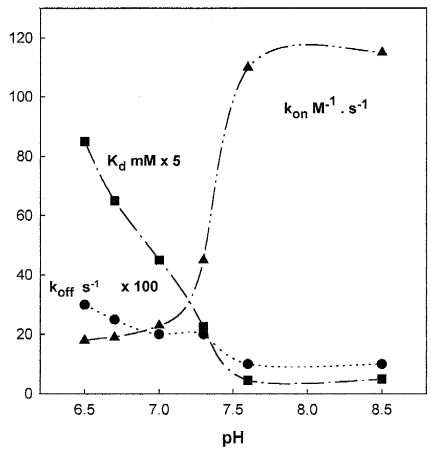

Fig. 4. pH-dependencies of binding constants of cyanide to ferrous HRP. Fully reduced, cyanide-ligated HRP was prepared by reduction with dithionite at pH 8.5 or with deazaflavin at pH 6.5 to pH 8.5, as described in Section 2 and photolysis and recombination of cyanide was monitored at 432–448 mm. At each pH, transients were taken at different concentrations of cyanide and k_{on}, k_{off} and K_d (k_{off}/k_{on}) were determined as described in Fig. 1.

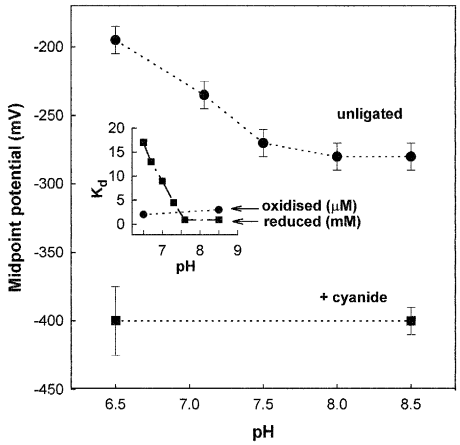

Fig. 5. Redox titrations of unligated and cyanide-ligated HRP. Anaerobic redox titrations of 2 μM HRP in 2.5 ml of 0.1 M potassium phosphate at different pH values were performed as described in Section 2. Heme reduction at each potential was assessed by a triple wavelength measurement at $440-(430+450)/2$ nm. The inset shows the pH-dependencies of the K_d of cyanide-ligated forms of oxidised and reduced HRP. The K_d values for reduced HRP were from Fig. 4 and those for the ferric form were estimated by titration of the optical change due to formation of the oxidised cyanide-ligated HRP at 426–400 nm.

3.4. pH-dependency of the kinetics parameters of cyanide recombination after flash photolysis

By use of both dithionite and the deazaflavin photoreduction system, it was possible to study cyanide photolysis and recombination to ferrous HRP in the range of pH from 6.5 to 8.5 without interference from redox changes. Values for k_{on}, k_{off} and K_d were determined from plots of k_{obs} versus cyanide concentration, as described in Fig. 1. The rate constants and K_d were pH-independent between pH 8.5 and 7.6. Below pH 7.5, K_d and k_{off} increased with decreasing pH. Between pH 7.5 and pH 7, the k_{on} decreased sharply from its high pH value of 120 M^{-1} s^{-1} and again became pH-independent at around 15 M^{-1} s^{-1} below pH 7 (Fig. 4).

3.5. pH-dependency of the midpoint potential of the cyanide-ligated enzyme

Potentiometric titrations of the unligated and cyanide-ligated forms of HRP are presented in Fig. 5. The behaviour of the unligated HRP is already well known and shows a characteristic -60 mV/pH dependency at low pH with a pK on the reduced form around pH 7.5 [26]. In contrast, the midpoint potential of the cyanide-ligated form was lowered to -400 mV and pH-independent between pH 6.5 and pH 8.5.

At saturating cyanide concentrations, the shift in midpoint potential induced by the binding of cyanide is expected to be 60 mV per 10-fold difference in the affinity of cyanide for oxidised compared to reduced forms [31]. The dissociation constants, K_d, of the cyanide ligated ferric and ferrous forms were determined by titration with cyanide of the magnitude of the binding spectra. These are plotted in Fig. 5, inset. As shown in Fig. 4, the binding of cyanide on the reduced enzyme was pH-dependent. A K_d of 0.9 mM was obtained at pH 8.5 and a K_d of 17 mM, at pH 6.5. In contrast, the K_d of 2–3 μM of the ferric-cyanide form was pH independent, in agreement with published values [32]. The observed midpoint potential shifts on binding of cyanide was therefore in agreement with these K_d values.

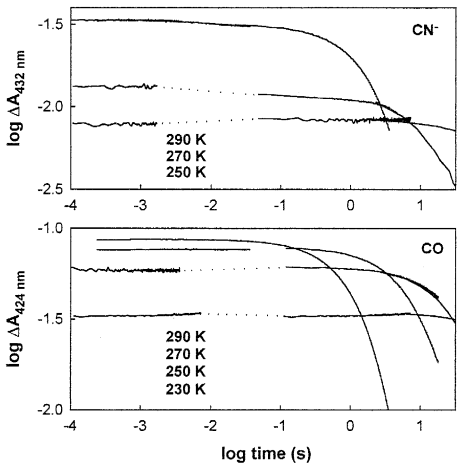

Fig. 6. Effects of temperature on recombination rates and photolysis yields of cyanide and CO. HRP was dissolved in 2 ml of 75% glycerol/25% potassium phosphate(v/v) at pH 8.5 to a final concentration of 2.4 μM. The sample was reduced with solid dithionite and either 10 mM potassium cyanide was added or the sample was saturated with CO. Laser-induced transients were recorded after equilibration at different temperatures. Photolysis and recombination were monitored at 432 nm for cyanide or 424 nm for CO. The data are plotted as log ΔA versus log time.

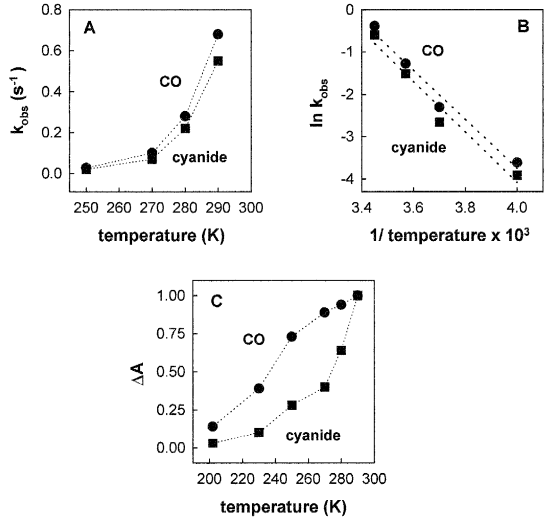

Fig. 7. Effects of temperature on photolysis yields and rates of recombination of cyanide and CO. The data of Fig. 6 are replotted to show the observed rates of recombination versus temperature (panels A and B) and the normalised photolysis yields versus temperature (panel C). At 292 K, the photolysis yields were 15% for cyanide and 30% for CO.

3.6. Effect of temperature on photolysis yield and rate of recombination

In Fig. 6, the extent of the transient at 432 nm at different temperatures has been plotted versus time in log/log form. Equivalent data for photolysis and recombination of the CO compound are given for comparison and are similar to data already presented elsewhere [21]. In the temperature range between 250 and 290 K and after the fastest time point of recording (in Fig. 6, this is 100 μs, although additional data were recorded to 1 μs without noticeable further changes), only the slow recombination from solution was observed, and no geminate processes are observable.

In Fig. 7, the photolysis yields and recombination rate constants derived from these same data have been plotted versus temperature. The effect of temperature on the observed rate constants of recombination was similar for both ligands (Fig. 7A). These data are replotted as an Arrhenius plot in Fig. 7B, and from the slopes fitted by least squares analyses activation energies of 48 and 50 kJ mol^{-1} may be calculated for CO and cyanide recombination, respectively. In contrast to the similarity of the temperature dependencies of these rate constants, the photolysis yield of cyanide was much more dramatically affected by temperature than that of CO (Fig. 7C).

4. Discussion

4.1. Formation of cyanide-ligated ferrous HRP

The cyanide anion is a well-known ligand of pentacoordinated heme iron. It binds more strongly to ferric than to ferrous heme because of the higher net positive charge of the former, and is a particularly strong ligand to ferric heme in accordance with its position in the spectrochemical series. However, because ferrous heme is uncharged overall, cyanide binding to this form is generally much weaker. In several cases, for example in ferrous hemoglobin [33] and ferrous myoglobin [22], the affinity for cyanide is extremely weak (dissociation constants are of the order of 1 M [33] and 0.4 M [22], respectively) so that ferrous cyanide compounds can only be observed at very high cyanide concentrations [30]. We have recently observed that the flavohemoglobins found in *Escherichia coli* [34–36] and in yeast [37,38] and cytochrome *bd* [39] also have large dissociation constants for cyanide from their reduced forms of ≥ 0.5 M (Meunier, B. and Rich, P.R., unpublished data). In contrast, the affinity of ferrous horseradish peroxidase for cyanide is much greater [30]. A dissociation constant in the mM range at alkaline pH has been reported [23], a value similar to that found in cytochrome-*c* peroxidase [40], cytochrome-*c* oxidase [41,42] and cytochrome *bo* [27]. Our data on the binding of added cyanide and on the recombination after photolysis confirm the literature value for alkaline conditions [23].

4.2. Redox- and ligand-linked protonation changes in HRP

The pH-dependency of the midpoint potential of HRP is already well known [26]. At acid pH a redox-linked proton is bound on reduction. However, the pK on the ferrous enzyme of around 7.5 for this protonation results in pH-independency at more alkaline values. At pH values higher than those considered here, further pH-dependency of midpoint poten-

tial is observed in association with the alkaline transition [26].

The pH-dependency of the dissociation constant of the cyanide compound of ferric HRP has been studied in detail [32] and shows clearly that the stable bound species involves the net binding of HCN, rather than CN^-, at least down to fairly low pH values. It has also been shown that the K_d of binding of cyanide to reduced HRP is independent of pH above pH 7.5 [23], again consistent with HCN being the net species bound. Below pH 7.5, however, the K_d increases with decreasing pH (Fig. 4). This suggests that when cyanide binds to the protonated ferrous HRP (below pH 7.5), the redox-linked proton prevents an additional proton being bound in association with the cyanide. Hence, between pH 6.5 and 8.5 the ferrous HRP-cyanide remains unchanged in its protonation state. A previous suggestion that this state displays a pH-dependent spectrum [43] is likely to be artefactual and caused by a degree of oxidation at low pH caused by an insufficient reducing potential generated by dithionite as we have shown in Figs. 2 and 3.

The transition of k_{on} caused by pH between its pH-independent values is rather sharp (Fig. 4), suggesting that more than one protonatable group could be involved. However, these data quality are not sufficient to quantitate the value with confidence. Instead, this same value may be deduced more reliably from the pH-dependency of midpoint potential (Fig. 5 and [26]) and this cleary indicates that only a single protonatable group on the ferrous enzyme is involved.

The mutual exclusivity of the redox- and cyanide-linked protonations is further corroborated by the pH-independency of the midpoint potential of cyanide-ligated HRP (Fig. 5) which indicates that the binding of cyanide and its associated proton prevents a redox-linked protonation.

4.3. Possible protonation sites

Two possibilities may account for the mutual exclusivity of the redox- and cyanide-linked protonations: the protons might compete for the same protonation site or, alternatively, the two sites might be structurally separate, but interact so that the binding of a proton in one site shifts the pK of the other site.

Although there has been considerable discussion of possible protonation sites in the peroxidases, the matter appears to be far from resolved. In both HRP and C*c*P, the distal histidine has been proposed to provide the protonation site for the redox-linked proton [21,44–47]. In HRP, protonation of the site is responsible for the pK_{red} of around 7.5 [26], for a small change in recombination rate constant with CO [18], and for spectral shifts in the visible band [44], Raman [45,47], infrared [48] and NMR [49] spectra. However, the identity of this protonation site with His-42 has been challenged recently [50,51]. Other possibilities for the protonation sites are provided by Arg-38 and its associated water molecules [25], by the heme propionates and their associated amino acids (as is the case for many *c*-type cytochromes which exhibit redox-linked pK shifts [31,52,53]), or by other protonatable groups which may be influenced by the heme redox state, such as His-40. However, the proximal histidine itself can be ruled out as a possible protonatable site since it is hydrogen-bonded to Asp-247, and both Raman [45,47,54] and NMR [49,55] data show that it does not change its protonation state with pH.

In the case of C*c*P, the situation is more complex since it has been shown that analogous changes at roughly the same pK are associated with a concerted two-proton change [56,57]. One of the protonation sites has been identified by site-directed mutagenesis as His-181, a residue which is hydrogen-bonded to a heme propionate [58]. This histidine is not conserved in HRP [59].

The situation regarding the site of anion-linked protonation is clearer. Crystal structure studies of the cyanide and fluoride complexes of oxidised C*c*P show clear hydrogen bonding to, and therefore protonation of, the distal histidine residue [60,61]. This conclusion is corroborated by Raman [45,47], infrared [62] and NMR [49] studies of the cyanide complex of ferric HRP.

Our data showing the exclusivity of the redox- and anion-linked protonation sites strongly suggests to us that the primary site in both cases in HRP is indeed provided by the distal histidine residue. Presumably, the distal histidine in C*c*P behaves in the same way, but is co-operatively linked to protonation changes at His-181. There is no evidence for a similar linkage to a group associated with the heme propionates in HRP

[63], although this may be a point to test further experimentally, particularly with the availability of mutant enzyme forms and when the atomic structure around these groups is solved.

We have provided some evidence from studies of HRP mutants of Arg-38 and His-42 that the distal arginine and associated water might provide an additional site needed for cyanide binding to the ferrous heme below pH 7 [25]. However, the present data indicate that two simultaneous protonations do not occur. Nevertheless, charge compensation in some form is required for concurrent reduction and cyanide anion binding and the more limited charge-compensating possibilities are still likely to be a major reason for the more dramatic decrease in affinities of the ferrous, compared to the ferric, forms of the mutants for cyanide. Further studies of the pH-dependencies of the midpoint potentials of these mutants is underway to shed further light on these effects.

4.4. Photolysis properties

Keilin and Hartree [30] first noted the photolability of the cyanide adduct of ferrous HRP. However, photochemistry of cyanide-ferroheme compounds is much more poorly studied than that of analogous CO compounds. In the case of myoglobin, the very high dissociation constant of its cyanide compound is prohibitive. The millimolar range K_d of the cyanide compound of ferrous HRP makes it far more experimentally accessible [24]. Of particular interest in the HRP system is the fact that photolysis of the cyanide into solution at alkaline pH should be accompanied by the co-migration of a proton [25], hence allowing the study of laser-induced proton photolysis.

We show here that the stable photolysis spectra, taken 100 ms and later after photolysis, correspond exactly to an inverse of the static binding spectrum. Furthermore, the recombination rates increase linearly with the concentration of cyanide in solution. Hence, it can be concluded that the processes that we are observing at room temperature are a simple photodissociation into solution to generate a normal unligated ferrous state, followed by a simple single-step recombination without any significant occupation of a prebinding step.

Subnanosecond geminate recombination of CO to HRP at room temperature [20], and in μs at low temperatures [21], has already been demonstrated. The fact that the yield of photolysis of the cyanide compound is low when using the laser pulse is likely to arise from a rapid geminate recombination process which is too fast (Fig. 6 shows data to 100 μs and further data take this limit to 1 μs) to measure in our experiments. An increased yield of cyanide photolysis on using the longer flash may then arise simply by repeated photolysis of material which has recombined geminately within the lifetime of the long flash.

As it has been shown for the CO compound, the photolysis yield of the ferrous cyanide compound was temperature-dependent (Fig. 7). However, the cyanide photolysis yield decreased more dramatically with lower temperature, possibly reflecting a strongly temperature-dependent step of cyanide dissociation into solution after initial formation of a geminate state [21]. This seems reasonable if HCN has to reform before dissociation into solution can occur. In contrast, the temperature dependency of recombination of cyanide from solution was roughly the same as that exhibited by CO, with an activation energy in both cases of around 50 kJ mol^{-1}.

In summary, we show here the mutual interplay between redox- and anion-linked protonation processes in HRP and present the first detailed characterisation of photolysis of the ferroheme-cyanide compound. Conditions have been identified in which cyanide photolysis will be accompanied by the co-migration of a proton, presumably from the distal histidine in the heme pocket. The system therefore provides a means for study not only of movement of a more hydrophilic ligand through the protein structure, but also its associated co-migrating proton. The questions of whether the immediate photolysed species must be HCN or the cyanide anion, and of whether the protonation changes needed to restore equilibrium occur after the initial photolysis events, remain to be established.

Acknowledgements

The authors would like to thank Prof. V. Massey for his kind gift of deazaflavin. This work is funded by a European Community fellowship award to B.M.

and by a Engineering and Physical Science Research Council research grant to P.R.R. (Grant GR/J28148).

References

[1] Austin, R.H., Beeson, K., Eisenstein, L., Frauenfelder, H., Gunsalus, I.C. and Marshall, V.P. (1973) Science 181, 541–542.
[2] Austin, R.H., Beeson, K.W., Eisenstein, L., Frauenfelder, H. and Gunsalus, I.C. (1975) Biochemistry 14, 5355–5373.
[3] Chatfield, M.D., Walda, K.N. and Magde, D. (1990) J. Am. Chem. Soc. 112, 4680–4687.
[4] Traylor, T.G., Magde, D., Taube, D.J., Jongeward, K.A., Bandyopadhyay, D., Luo, J. and Walda, K.N. (1992) J. Am. Chem. Soc. 114, 417–429.
[5] Petrich, J.W., Poyart, C. and Martin, J.L. (1988) Biochemistry 27, 4049–4060.
[6] Taube, D.J., Projahn, H., van Eldik, R., Magde, D. and Traylor, T.G. (1990) J. Am. Chem. Soc. 112, 6880–6885.
[7] Elber, R. and Karplus, M. (1990) J. Am. Chem. Soc. 112, 9161–9175.
[8] Buhks, E. and Jortner, J. (1985) J. Chem. Phys. 83, 4456–4462.
[9] Balasubramanian, S., Lambright, D.G., Marden, M.C. and Boxer, S.G. (1993) Biochemistry 32, 2202–2212.
[10] Welinder, K.G. (1992) Curr. Opin. Struct. Biol 2, 388–394.
[11] Finzel, B.C., Poulos, T.L. and Kraut, J. (1984) J. Biol. Chem. 259, 13027–13036.
[12] Edwards, S.L., Raag, R., Wariishi, H., Gold, M.H. and Poulos, T.L. (1993) Proc. Natl. Acad. Sci. USA 90, 750–754.
[13] Schuller, D.J., Ban, N., Vanhuystee, R.B., McPerson, A. and Poulos, T.L. (1996) Structure 4, 311.
[14] Smulevich, G., Paoli, M., Burke, J.F., Sanders, S.A., Thorneley, R.N.F. and Smith, A.T. (1994) Biochemistry 33, 7398–7407.
[15] Rodriguez-Lopez, J.N., Smith, A.T. and Thorneley, R.N.F. (1995) J. Biol. Chem. 271, 4023–4030.
[16] Gilfoyle, D.J., Rodriguez-Lopez, J.N. and Smith, A.T. (1996) Eur. J. Biochem. 236, 714–722.
[17] Kertesz, D., Antonini, E., Brunori, M., Wyman, J. and Zito, R. (1965) Biochemistry 4, 2672–2676.
[18] Coletta, M., Ascoli, F., Brunori, M. and Traylor, T.G. (1986) J. Biol. Chem. 261, 9811–9814.
[19] Brunori, M., Giacometti, G.M., Antonini, E. and Wyman, J. (1973) Proc. Natl. Acad. Sci. USA 70, 3141–3144.
[20] Berinstain, A.B., English, A.M., Hill, B.C. and Sharma, D. (1990) J. Am. Chem. Soc. 112, 9649–9650.
[21] Doster, W., Bowne, S.F., Frauenfelder, H., Reinisch, L. and Shyamsunder, E. (1987) J. Mol. Biol. 194, 299–312.
[22] Bellelli, A., Antonini, G., Brunori, M., Springer, B.A. and Sligar, S.G. (1990) J. Biol. Chem. 265, 18898–18901.
[23] Phelps, C., Antonini, E. and Brunori, M. (1971) Biochem. J. 122, 79–87.
[24] Hill, B.C. and Marmor, S. (1991) Biochem. J. 279, 355–360.
[25] Meunier, B., Rodriguez-Lopez, J.N., Smith, A.T., Thorneley, R.N.F. and Rich, P.R. (1995) Biochemistry 34, 14687–14692.
[26] Harbury, H.A. (1957) J. Biol. Chem. 225, 1009–1024.
[27] Mitchell, R., Moody, A.J. and Rich, P.R. (1995) Biochemistry 34, 7576–7585.
[28] Massey, V. and Hemmerich, P. (1977) J. Biol. Chem. 252, 5612–5614.
[29] Mayhew, S.G. (1978) Eur. J. Biochem. 85, 535–547.
[30] Keilin, D. and Hartree, E.F. (1955) Biochem. J. 61, 153–171.
[31] Moore, G.R. and Pettigrew, G.W. (1990) Springer-Verlag, Berlin.
[32] Ellis, W.D. and Dunford, H.B. (1968) Biochemistry 7, 2054–2062.
[33] Brunori, M., Antonini, G., Castagnola, M. and Bellelli, A. (1992) J. Biol. Chem. 267, 2258–2263.
[34] Orii, Y., Ioannidis, N. and Poole, R.K. (1992) Biochem. Biophys. Res. Commun. 187, 94–100.
[35] Ioannidis, N., Cooper, C.E. and Poole, R.K. (1992) Biochem. J. 288, 649–655.
[36] Andrews, S.C., Shipley, D., Keen, J.N., Findlay, J.B.C., Harrison, P.M. and Guest, J.R. (1992) FEBS Lett. 302, 247–252.
[37] Oshino, R., Asakura, T., Takio, K., Oshino, N., Chance, B. and Hagihara, B. (1973) Eur. J. Biochem. 39, 581–590.
[38] Zhu, H. and Riggs, A.F. (1992) Proc. Natl. Acad. Sci. USA 89, 5015–5019.
[39] Poole, R.K., D'Mello, R., Hill, S., Ioannidis, N., Leung, D. and Wu, G. (1994) Biochim. Biophys. Acta 1187, 226–231.
[40] Erman, J.E. (1974) Biochemistry 13, 39–44.
[41] Van Buuren, K.J.H., Nicholls, P. and van Gelder, B.F. (1972) Biochim. Biophys. Acta 256, 258–276.
[42] Antonini, E., Brunori, M., Greenwood, C., Malmström, B.G. and Rotilio, G. (1971) Eur. J. Biochem. 23, 396–400.
[43] Yamada, H. and Yamazaki, I. (1975) Arch. Biochem. Biophys. 171, 737–744.
[44] Yamazaki, I., Araiso, T., Hayashi, Y., Yamada, H. and Makino, R. (1978) Adv. Biophys. 11, 249–281.
[45] Kitagawa, T. and Teraoka, J. (1982) The Biological Chemistry of Iron (Dunford, H.B., Dolphin, D., Raymond, K.N. and Sieker, L. eds.), pp. 375–389, D. Reidel Publishing Company, Dordrecht, Holland.
[46] Teraoka, J. and Kitagawa, T. (1980) Biochem. Biophys. Res. Commun. 93, 694–700.
[47] Teraoka, J. and Kitagawa, T. (1981) J. Biol. Chem. 256, 3969–3977.
[48] Barlow, C.H., Ohlsson, P.-I. and Paul, K.-G. (1976) Biochemistry 15, 2225–2229.
[49] Thanabal, V., De Ropp, J.S. and La Mar, G.N. (1988) J. Am. Chem. Soc. 110, 3027–3035.
[50] Holzbaur, I.E., English, A.M. and Ismail, A.A. (1996) J. Am. Chem. Soc., in press.
[51] Smulevich, G., Miller, M.A., Kraut, J. and Spiro, T.G. (1991) Biochemistry 30, 9546–9558.

[52] Gao, Y., McLendon, G., Pielak, G.J. and Williams, R.J.P. (1992) Eur. J. Biochem. 204, 337–352.
[53] Williams, R.J.P. (1991) Biochim. Biophys. Acta 1058, 71–74.
[54] Al-Mustafa, J. and Kincaid, J.R. (1994) Biochemistry 33, 2191–2197.
[55] La Mar, G.N., De Ropp, J.S., Chacko, V.P., Satterlee, J.D. and Erman, J.E. (1982) Biochim. Biophys. Acta 708, 317–325.
[56] Conroy, C.W., Tyma, P., Daum, P.H. and Erman, J.E. (1978) Biochim. Biophys. Acta 537, 62–69.
[57] Iizuka, T., Makino, R., Ishimura, Y. and Yonetani, T. (1985) J. Biol. Chem. 260, 1407–1412.
[58] Miller, M.A., Mauro, J.M., Smulevich, G., Coletta, M., Kraut, J. and Traylor, T.G. (1990) Biochemistry 29, 9978–9988.
[59] Welinder, K.G., Bjornholm, B. and Dunford, H.B. (1995) Biochem. Soc. Trans. 23, 257–262.
[60] Edwards, S.L. and Poulos, T.L. (1990) J. Biol. Chem. 265, 2588–2595.
[61] Edwards, S.L., Xuong, N.H., Hamlin, R.C. and Kraut, J. (1987) Biochemistry 26, 1503–1511.
[62] Yoshikawa, S., O'Keefe, D.H. and Caughey, W.S. (1985) J. Biol. Chem. 260, 3518–3528.
[63] Smith, A.T., Du, P. and Loew, G.H. (1995) Nuclear Magnetic Resonance of Paramagnetic Macromolecules (La Mar, G.N. ed.), pp. 75–93, Kluwer, Dordrecht.